AD_____

Award Number: W81XWH-06-1-0746

TITLE: Microtubule-Associated Protein Expression and Predicting Taxane Response

PRINCIPAL INVESTIGATOR: Maria T. Baquero

CONTRACTING ORGANIZATION: Yale University
New Haven, CT 06520-8023

REPORT DATE: October 2008

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
1 Oct 2008	Annual Summary	15 Sep 2007 – 14 Sep 2008

4. TITLE AND SUBTITLE

Microtubule-Associated Protein Expression and Predicting Taxane Response

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-06-1-0746

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

Maria T. Baquero

E-Mail: mt.baquero@yale.edu

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Yale University
New Haven, CT 06520-8023

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

We hypothesized that in addition to its predictive value, the microtubule-associated marker tau (MAP-tau) may also function as a prognostic biomarker. The dual functionality of MAP-tau may translate into increased tumor molecular screening information for patients with breast cancer resulting in better treatment options. Previous results of this work indicate that MAP-tau functions as a prognostic marker in early stage breast cancer when examined using tissue microarrays and automated quantitative analysis (AQUA). Current work corroborates these results in the metastatic setting as well using tissue whole sections from the TAX 307 clinical trial . This work demonstrates that increased MAP-tau expression in both early and metastatic settings is associated with better outcome and increased time to tumor progression, respectively. Our findings suggest that MAP-tau is a useful prognostic marker but is not predictive of taxane response. Future work will examine MAP-tau in combination with destabilizing MAPs such as stathmin which may provide a more robust model for taxane responsiveness.

15. SUBJECT TERMS
Microtubule-associated proteins (MAPs), MAP-tau, breast cancer, tissue microarrays, biomarkers

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
U	U	U	UU	29	19b. TELEPHONE NUMBER *(include area code)*

Table of Contents

Page

Introduction

Breast cancer is the leading cause of cancer death in women between the ages of 20 and 59 accounts for more than 31% of all new cancers diagnosed in women and is the leading cause of death for women worldwide [1, 2]. While breast cancer family history is an important risk factor, sporadic cases account for more than 90% of all breast cancers and the etiology of this cancer remains largely unknown [3]. Clinical treatment, such as chemotherapy, currently relies on physical examination, imaging, histopathological information, tumor size, lymph node status, degree of metastasis, and biomarker expression (ER, PR, HER2) [4] .

Microtubule stabilizing proteins, such as tau, have begun to gain attention as predictive markers. Tau expression has been found to decrease microtubule vulnerability to taxanes such as *paclitaxel* and its expression makes cells resistant to taxane treatment. Similarly it has recently been shown that low Tau is predictive for response to paclitaxel in breast cancer [5].

Current breast cancer therapy involves the use of taxanes such as paclitaxel and docetaxel [6]. Low tau expression has been shown to be predictive for response to paclitaxel in the neoadjuvant setting. However, the prognostic and predictive value of MAP-tau in early stage and metastatic breast cancer has not been established [5]. This study examined MAP-tau expression in relation to overall patient survival as well as the predictive value of MAP-tau in determining responsiveness to taxane treatment in early stage and metastatic breast cancer.

Body

In Aim 2 of this project, whole tumor sections from the TAX 307 clinical trial [52, 53], in collaboration with the laboratory of Dr. Lyndsay Harris, were developed as a training set to determine the predictive value of MAP-tau followed by the number of fields and qualification of fields necessary to discriminate low MAP-tau expression from high MAP-tau expression with statistical accuracy as well as biological relevance. For the evaluation of a MAP-tau AQUA score cutpoint, we predicted that using the second peak, reflecting the median AQUA score in the case of bimodal distributions of MAP-tau with continuous AQUA scores, and the last peak, in cases of multi-modal distributions, would most accurately reflect the biology of MAP-tau expression in the TAX 307 cohort.

We tested this hypothesis by performing fluorescence-based immunohistochemistry on 140 floated, whole tumor sections and 6 YTMA-94-1 control tissue microarrays containing cell lines MB468, BT474, ZR75.1, T47D, and MB231. These sections were accompanied by H&E slides with long term clinical follow up data from patients randomized to the AT arm of TAX 307. Automated quantitative analysis (AQUA) was used to evaluate MAP-tau expression, with cytokeratin staining defining pixels as breast cancer within the array spot and Cy-5 conjugated antibodies used to measured the intensity of MAP-tau expression. Image acquisition was limited to all tumor section

areas containing cytokeratin staining. Approximately 15,686 images were collected and analyzed due to the size of the tumor area and the image acquisition matrix utilized by PM2000 (Fig. 1). We found that MAP-tau showed a normal distribution of expression across six sets of tissue microarray cell lines (YTMA 94-1) (Fig.5) with high correlation (R= 0.84) (Fig. 5) indicating little batch staining variability. A distribution analysis of 15 randomly selected images was performed as a preliminary study to assess correlations between staining intensity and AQUA scores (Fig. 6).

Task 2: TAX 307 whole tissue sections with taxane treatment data will be used as a training cohort for future predictive markers

A retrospective cohort of patients treated with taxane therapy will be assembled and tissue samples from this cohort will be used to determine the predictive value of MAP-tau and to examine tissue heterogeneity.

The following items from the Statement of Work have been **completed:**

a. Select primary breast carcinoma tumors from the Yale Pathology archives or clinical trials that underwent taxane therapy.
   Completed: 140 whole tissue sections were obtained (Appendix B).

b. Design cell line controls for microtubule stabilizing proteins
   Completed: 6 YTMA 94-1 microarrays were stained to provide controls for whole tissue sections (Appendix C).

c. Analysis of whole tissue sections and tissue microarrays to examine tissue heterogeneity.
   Completed. 140 of the 140 whole tissue sections have been analyzed (Appendix D and E). Problems with some tissue loss due to whole sections being floated on the slides rather than previous use of tape-transfer method.

Timeline: Months 13-27

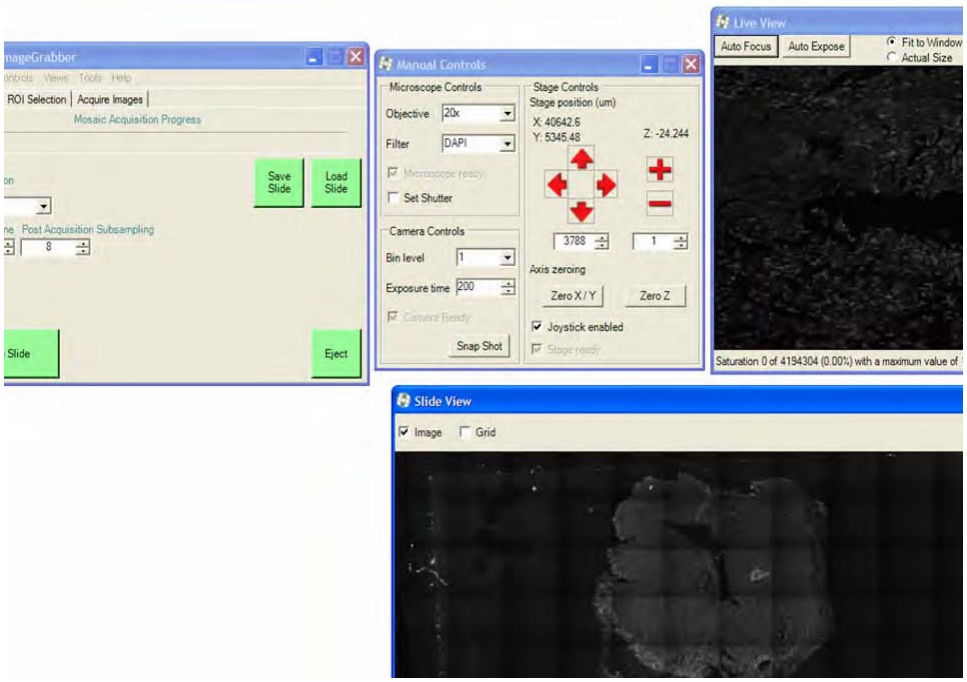
Task 3. Analysis of additional tubulin isoforms and other microbutuble stabilizing proteins as potential biomarkers for predicting response to taxanes.

a. Order breast test arrays and conduct antibody titration for each antibody using breast test arrays
   In Progress
b. Use standard clinical biomarker antibodies for immunofluorescence to stain the tissue microarrays with MAP-tau, E-MAP 115, MAP1A, MAP 1B, MAP2, MAP4, Dis1/TOG
   In Progress
c. Collect and validate images (350 histospots per slide, per antibody)
   In Progress

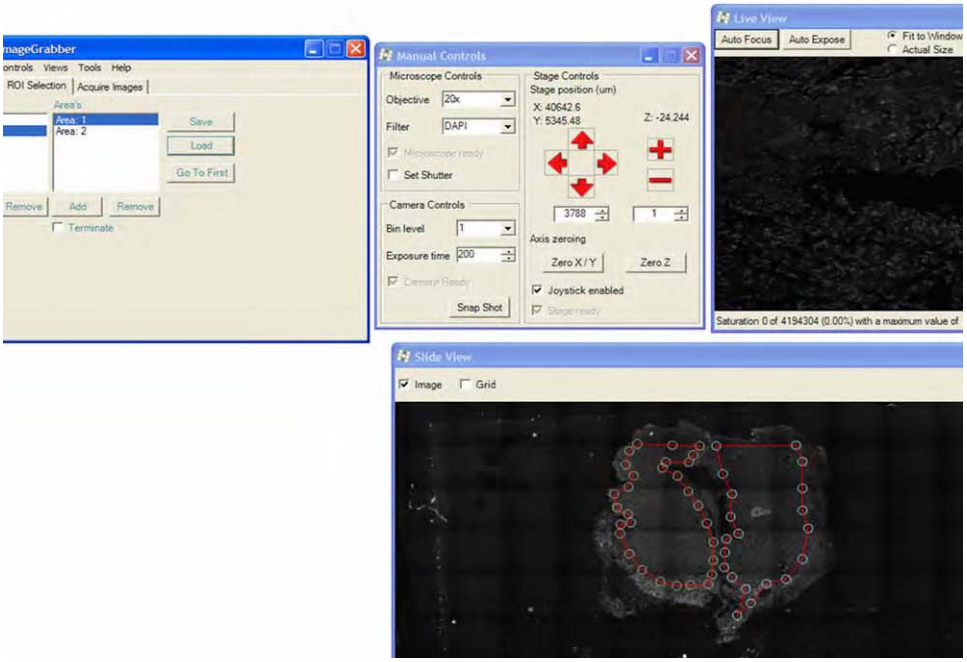
Timeline: Months 28-36 (2 months per antibody)

Image Capture for Whole Tissue Sections from TAX 307 Clinical Trial

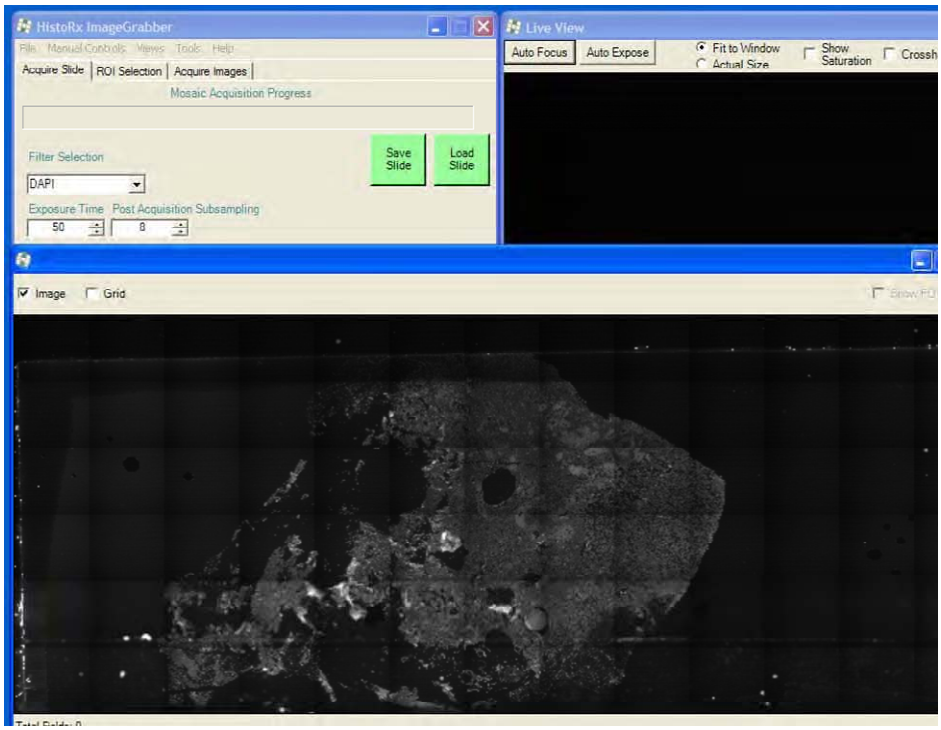
A.



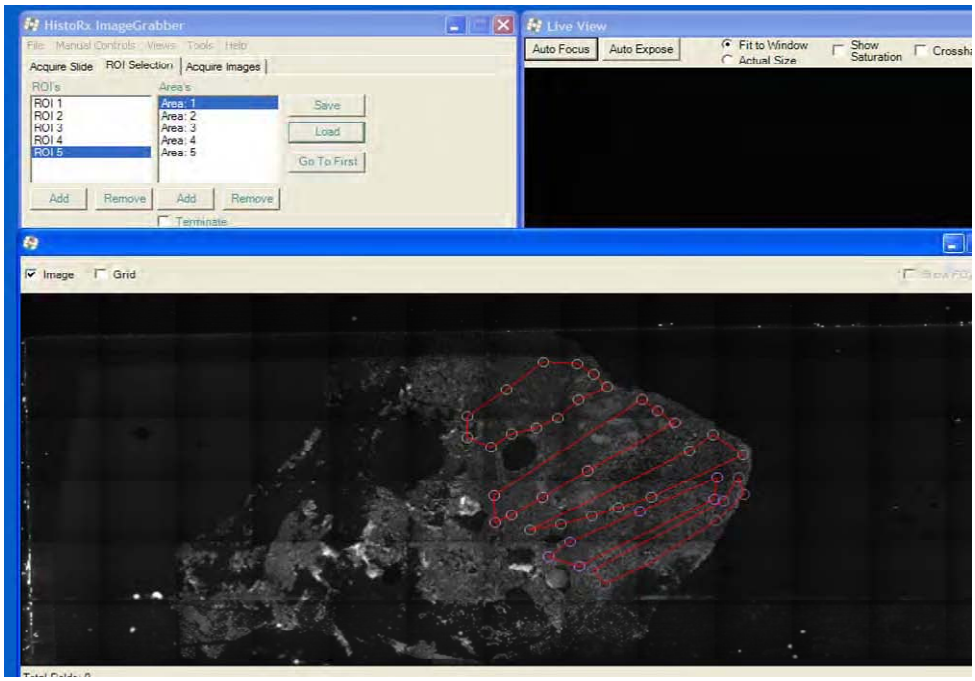
B.



C.


HistoRx ImageGrabber
File Manual Controls Views Tools Help
Acquire Slide
ROI Selection
Acquire Images
Mosaic Acquisition Progress
Filter Selection
DAPI
Exposure Time
Post Acquisition Subsampling
50
8
Save Slide
Load Slide
Live View
Auto Focus
Auto Expose
Fit to Window
Actual Size
Show Saturation
Image
Grid

D.


HistoRx ImageGrabber
File Manual Controls Views Tools Help
Acquire Slide
ROI Selection
Acquire Images
ROI's
ROI 1
ROI 2
ROI 3
ROI 4
ROI 5
Area's
Area: 1
Area: 2
Area: 3
Area: 4
Area: 5
Save
Load
Go To First
Add
Remove
Add
Remove
Terminate
Live View
Auto Focus
Auto Expose
Fit to Window
Actual Size
Show Saturation
Image
Grid

E.

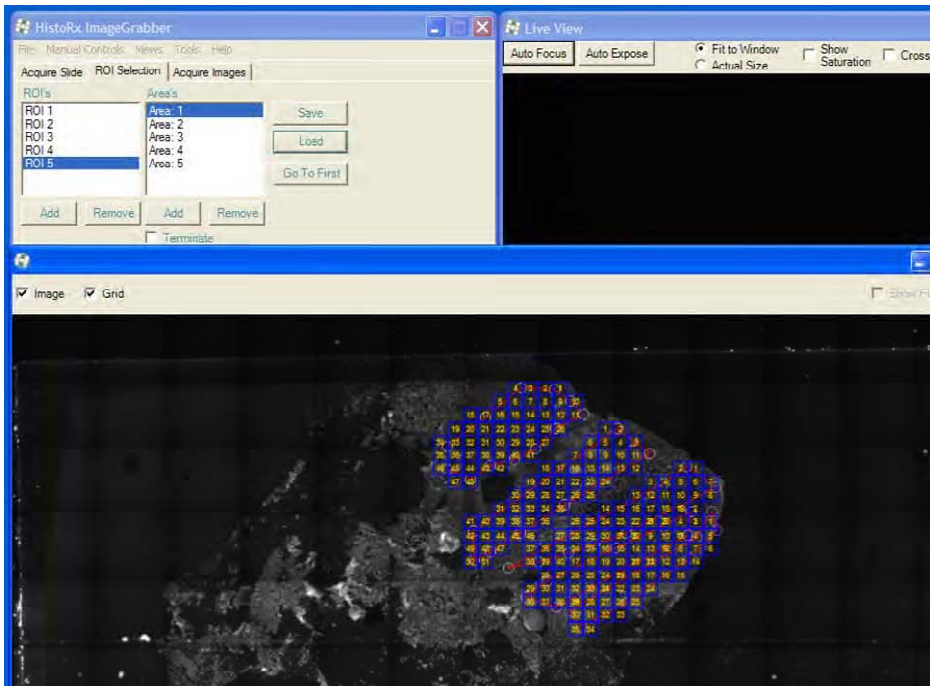


Fig 1. Whole tumor sections from two TAX307cases with image acquisition matrix circumscribing tumor area in red (bottom panels) and image acquisition software menus plus live image (top panel 1,2 and 3). A) Live view and low resolution image capture of whole tissue section from case 94 of TAX 307 using automated quantitative analysis and DAPI immunofluorescence. B) Matrix designation and acquisition order for low resolution image capture of whole tissue section from case 94 of TAX 307 using automated quantitative analysis and DAPI immunofluorescence. C) Live view and low resolution image capture of whole tissue section from case 81 of TAX 307 using automated quantitative analysis and DAPI immunofluorescence. D) Matrix designation for low resolution image capture of whole tissue section from case 81 of TAX 307 using automated quantitative analysis and DAPI immunofluorescence. E) Matrix designation and acquisition order for low resolution image capture of whole tissue section from case 81 of TAX 307 using automated quantitative analysis and DAPI immunofluorescence.

Image Capture Challenges:

- Only 61% of H&Es available
- Tissue distortion after staining: folding, tearing, shearing, erosion
- Sections not planar; Multiple ROIs
- First use 20x to "explore" tissue sections and plane of focus, then set ROIs Collapse any ROI into 2 if still out of focus
- PM3 makes many, many files! Label appropriately for patient cross-matching late
- Criteria for Excluding Cases (determined at beginning of study):
  1. No tissue on specimen slide
  2. Heavy tissue erosion: >95% of tissue gone
  3. Validation: out of focus, artifacts (uncropable)

Total Cases Excluded during image capture: 16
(16/140= 11.4% Excluded, 88.6% Retained)

Quantitative analysis of specimens:

Strengths:

- Rapid experiment run-time: ~11 TMA images per minute
- Tumor Histogram Threshold: 10-20% and can be adjusted per slide
- Relatively small template and analysis file size: <100 MB
- User-friendly interface with multiple ways to examine images

Limitations:

- No Cropping! so entire image must be discarded
- Still some bugs so error messages are common with too many open windows
- Exposure times may not register properly in manual mode

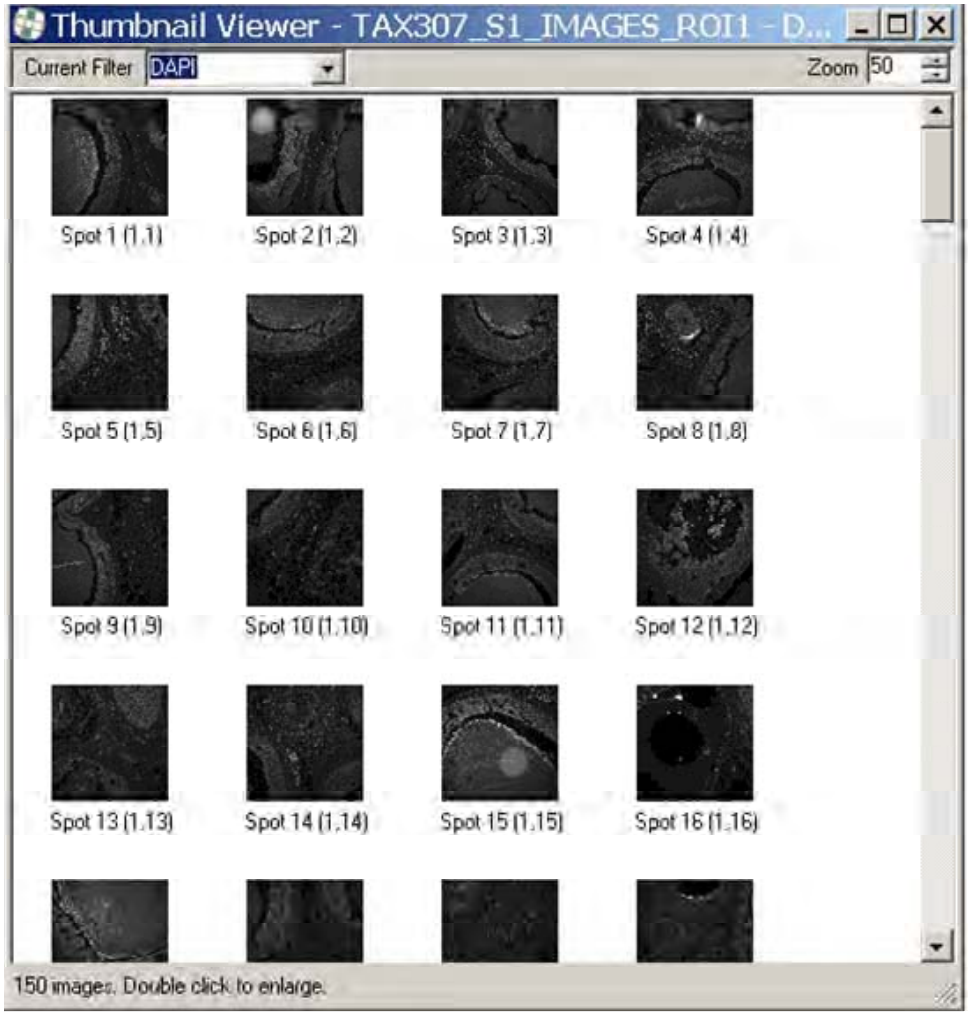
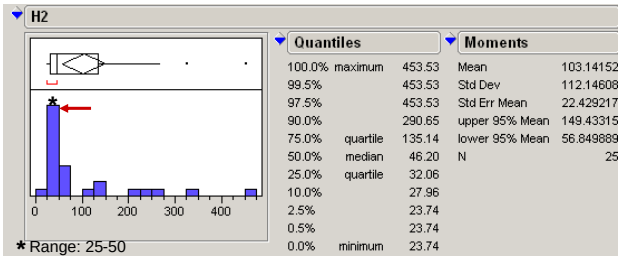


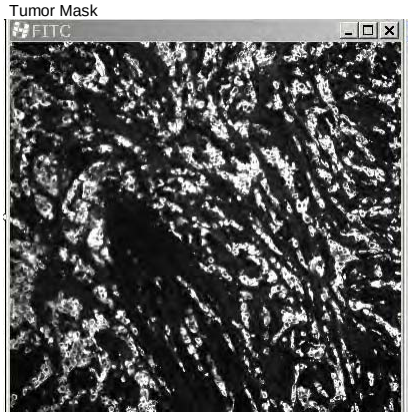
Fig. 2 Image thumbnails for case 1 after matrix designation and acquisition using automated quantitative analysis.

Case: H2.1

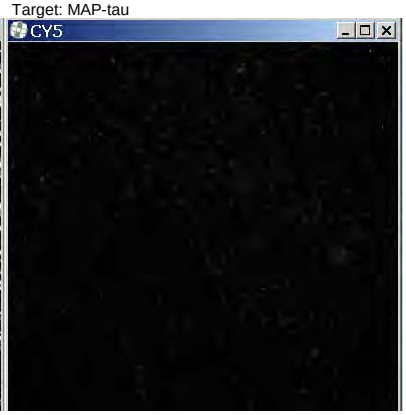
AQUA
Score: 40.68



Tumor Mask

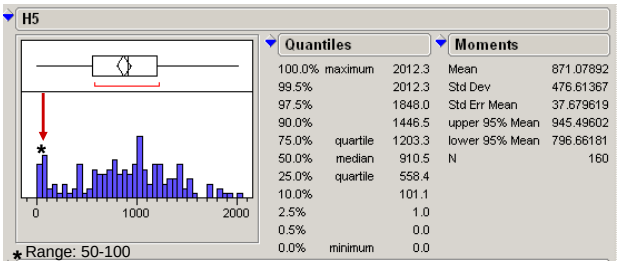


Target: MAP-tau

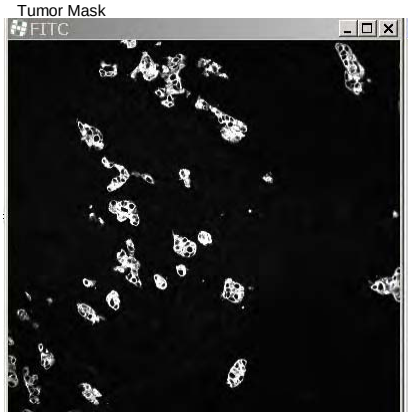


Case: H5.2

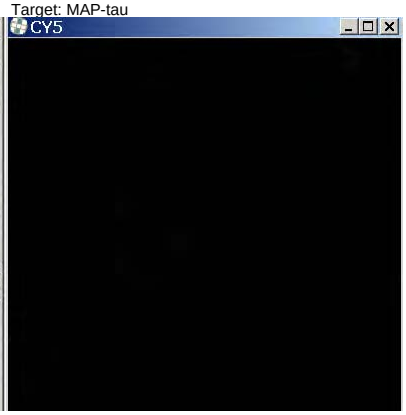
AQUA
Score: 63.77



Tumor Mask

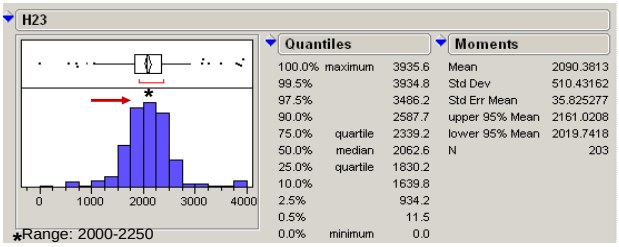


Target: MAP-tau

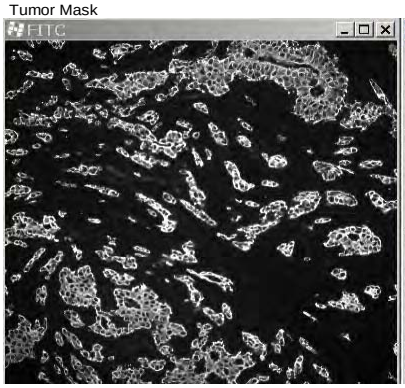


Case: H23.5

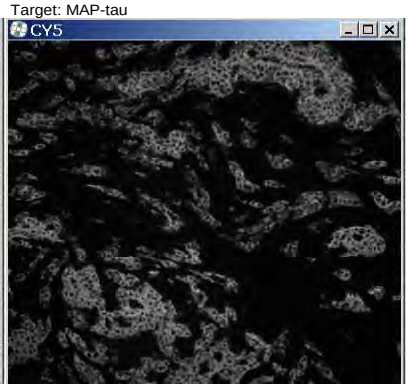
AQUA
Score: 2239.94



Tumor Mask

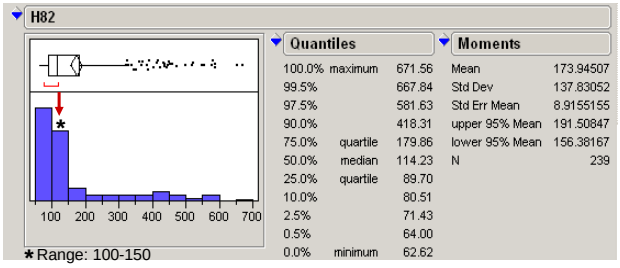


Target: MAP-tau

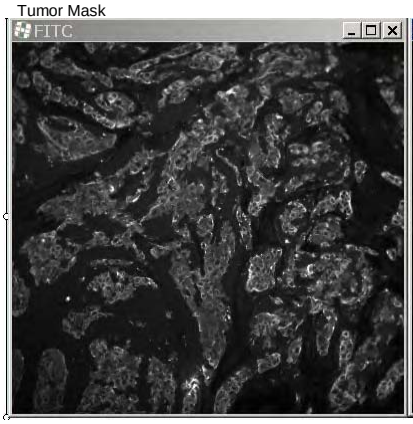


Case: H82.9

AQUA
Score: 105.16



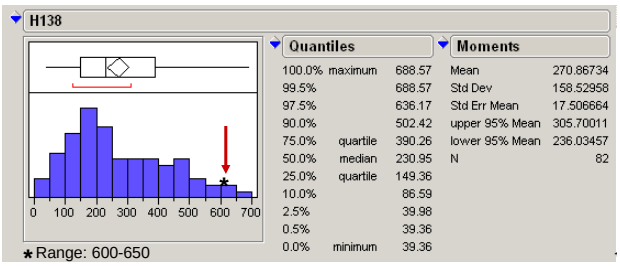
Tumor Mask



Target: MAP-tau

Case: H138.16

AQUA Score: 619.50



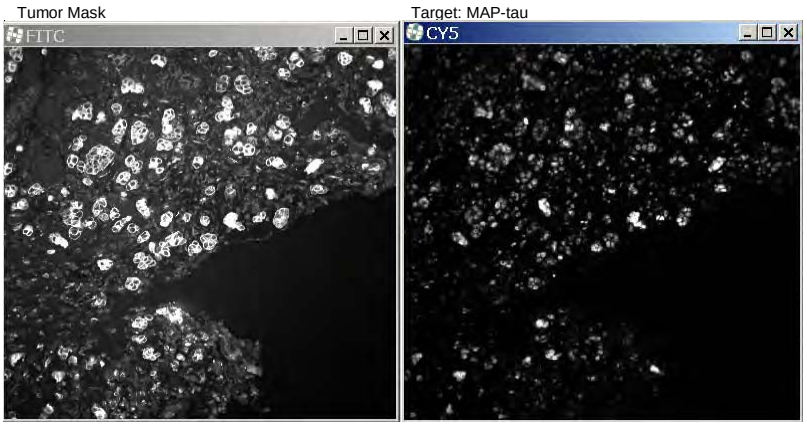


Fig. 3. TAX307 tumor section images and AQUA score for 5 selected cases. Distribution of AQUA scores for each case with red arrow representing current image AQUA score (top panel) and cytokeratin and MAP-tau staining intensity for tumor image visualized below (bottom two panels, respectively).

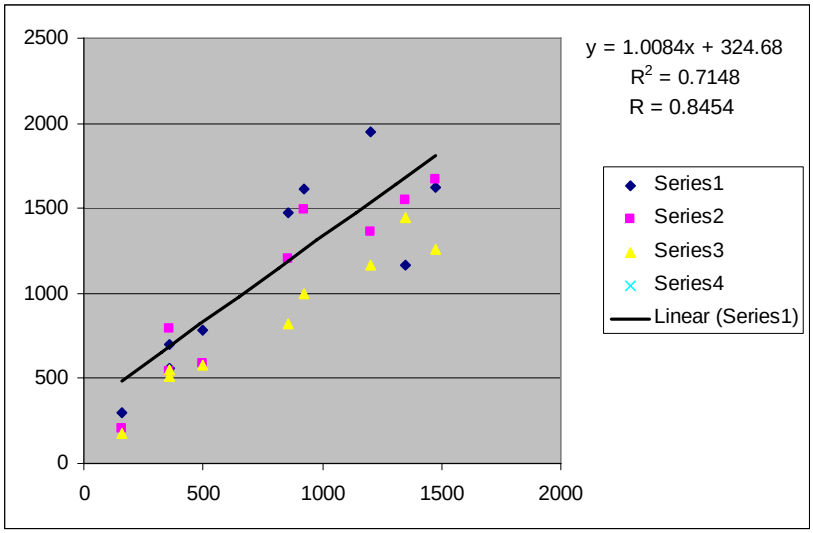


Fig. 4. TAX 307 cell line AQUA score regression for 94-1 arrays.

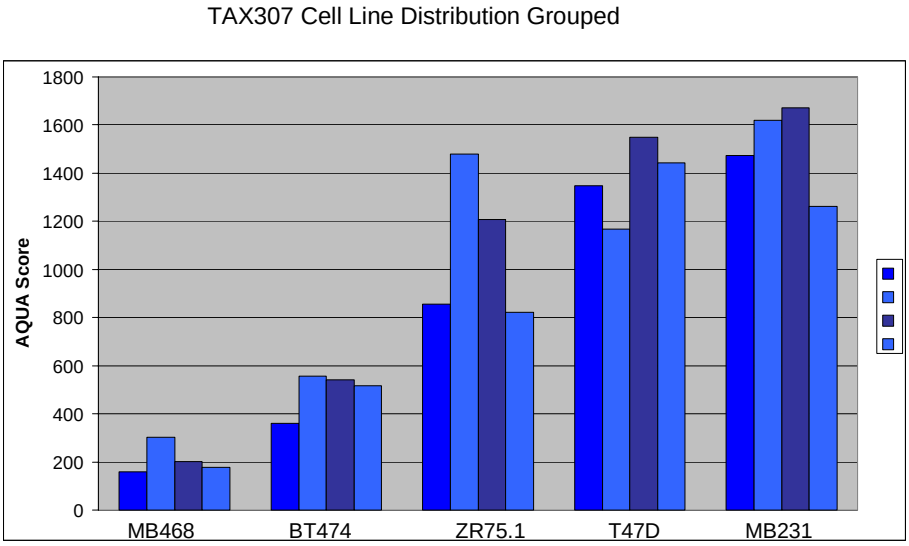


Fig. 5. TAX 307 cell line distributions.

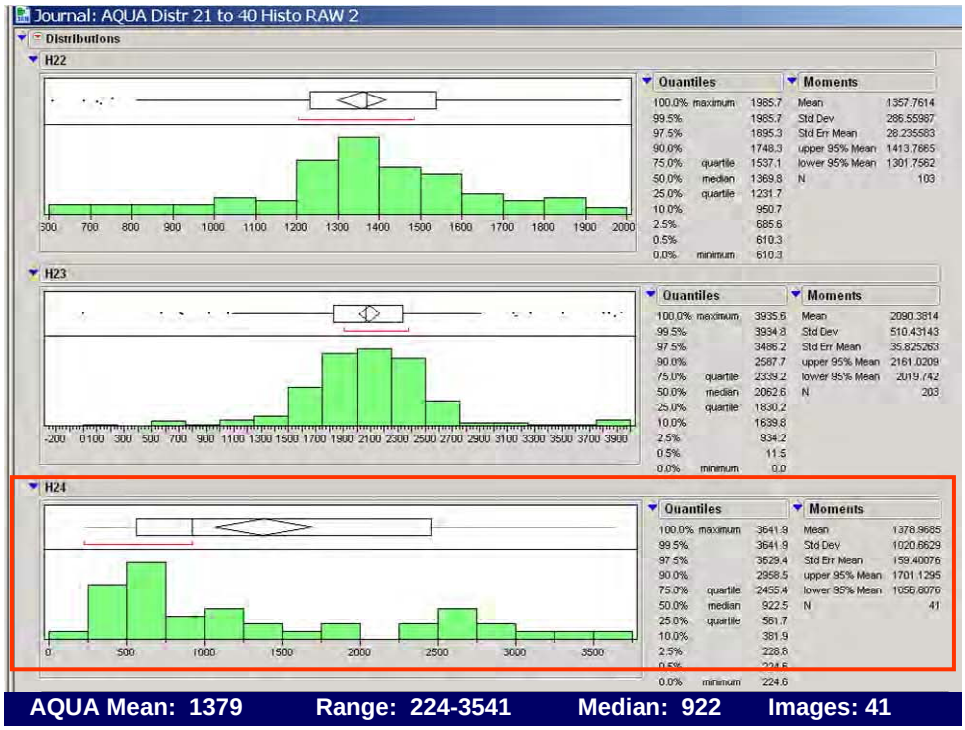
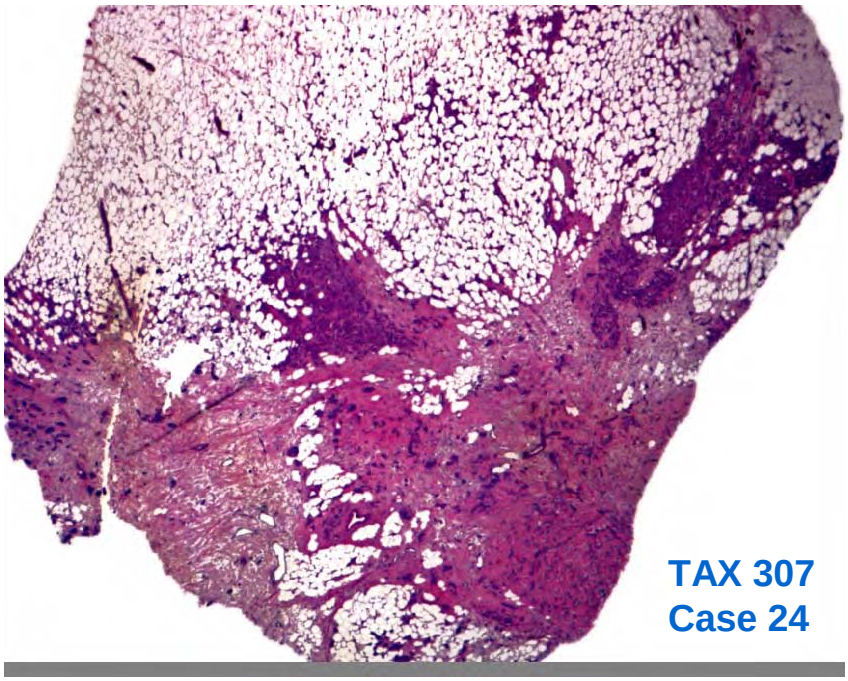
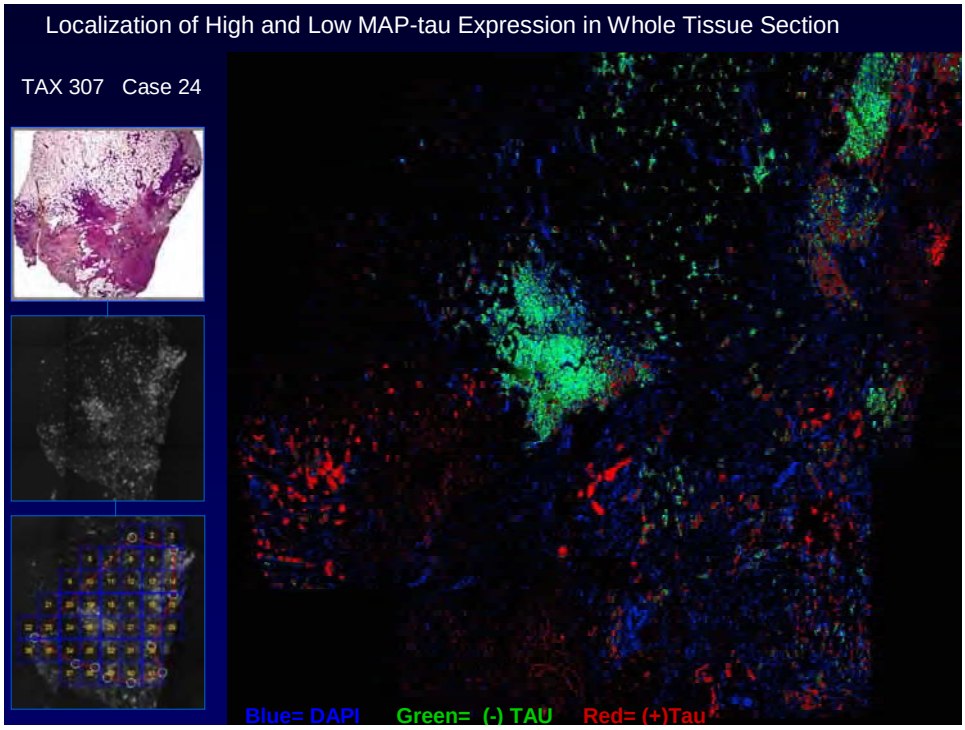


Fig. 6. Distribution analysis for three cases with mean, median, and quantile AQUA scores.

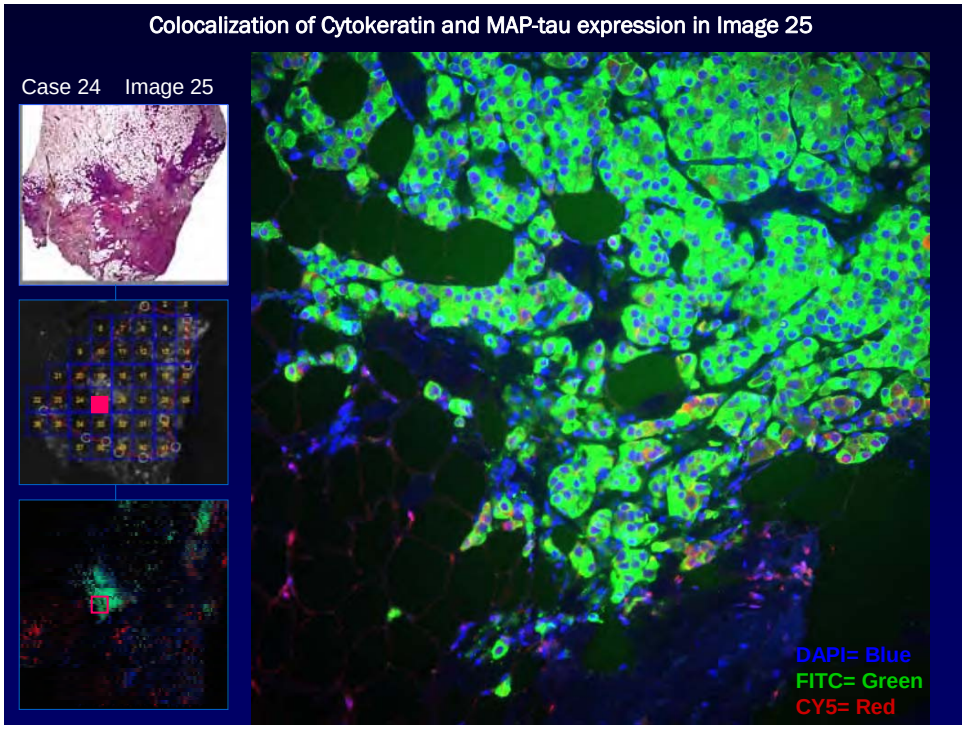
A.



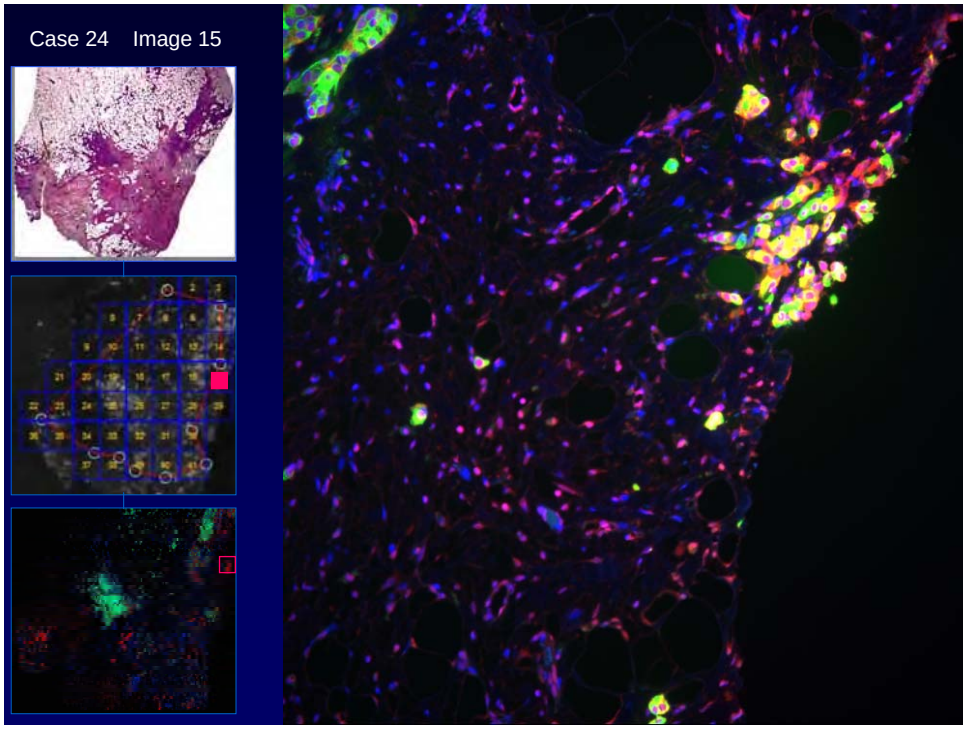
B.



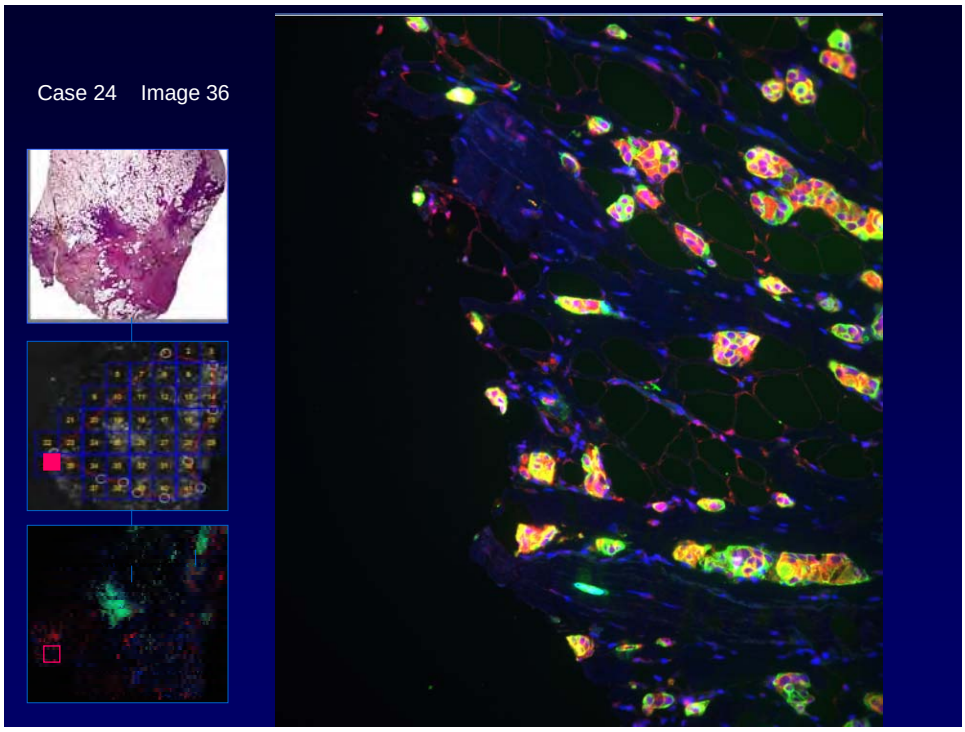
C.


Colocalization of Cytokeratin and MAP-tau expression in Image 25
Case 24 Image 25
DAPI= Blue
FITC= Green
CY5= Red

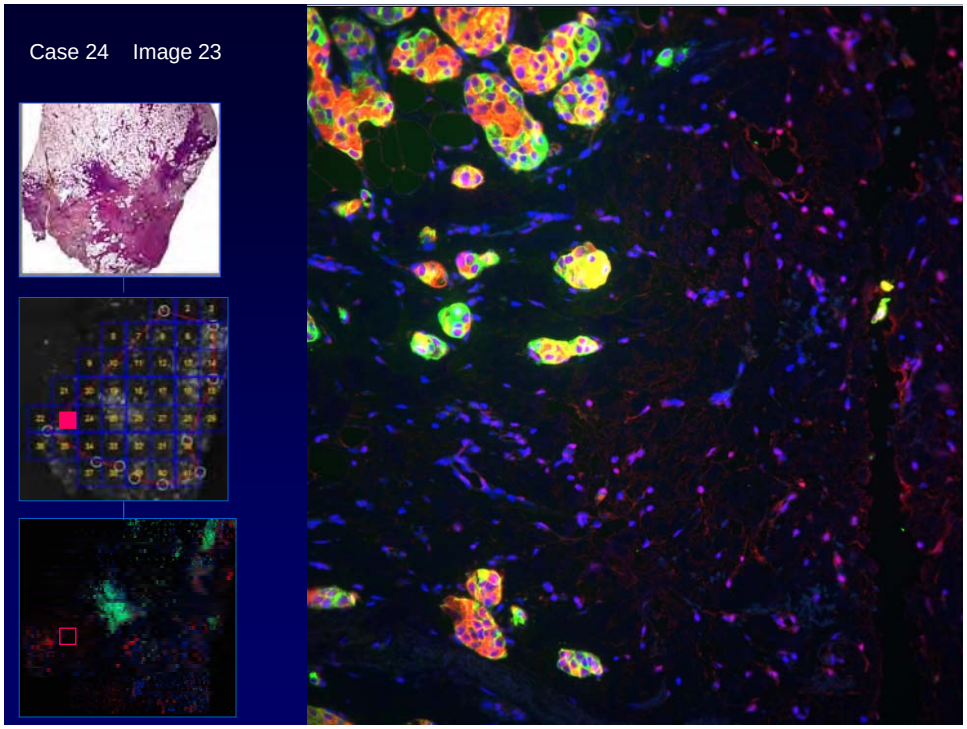
D.



E.



F.



G.

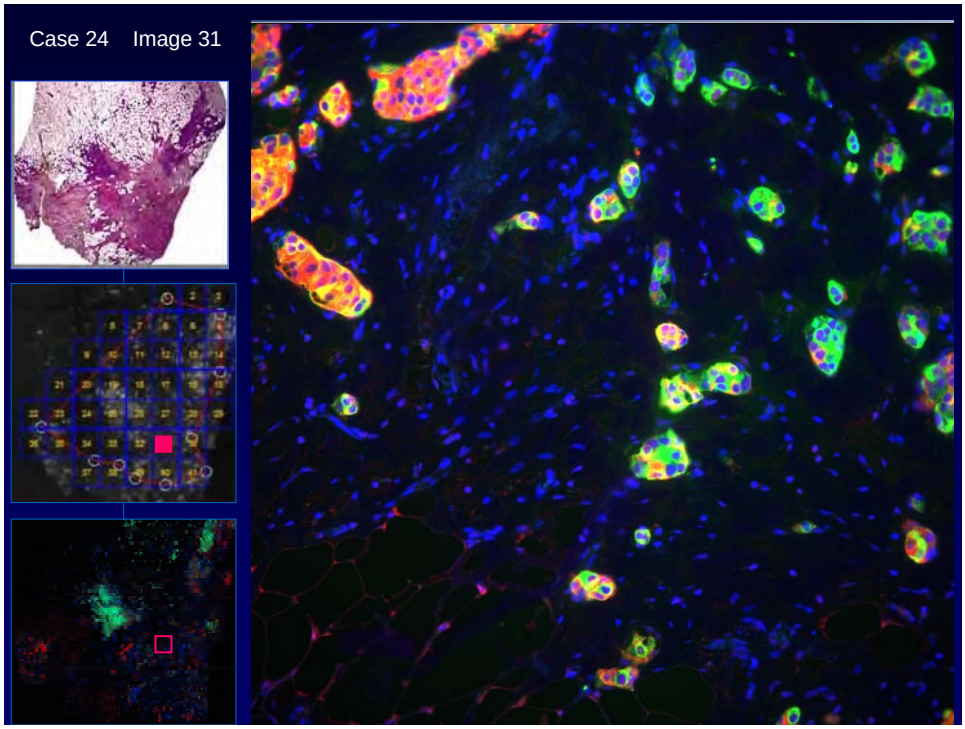


Fig. Case 24 with H&E (A) followed by high resolution image capture, matrix designation and acquisition order,and localization of low and high MAP-tau expression (B-G)

Key Research Accomplishments

- This work demonstrated that Tau functions as a prognostic marker for paclitaxel sensitivity using AQUA and the Tissue Arrays YTMA 49-5 and YTMA 49-6.
- Increased MAP-tau expression is associated with better outcome in breast cancer patients.
- MAP-tau may be a useful prognostic marker in addition to its predictive value for taxane response.
- Examining tissue heterogeneity using both whole tissue sections and tissue microarrays can provide important information regarding the usefulness of tissue microarrays in cancer diagnosis and treatment.

Reportable Outcomes

1. Era of Hope Breast Cancer conference abstract acceptance and poster presentation Baltimore, Maryland. June 2008. (Appendix D).
2. TAX 307 whole tissue sections stained with T1029 MAP-tau Mab (Appendix A and Figs. 1-5).
3. Whole Section Tissue database with 15, 604 images. (Figs. 1-5)
4. 6 control slides created: YTMA 94-1 tissue microarray with 120 histospots (Fig 4).
6. PhD dissertation research project that is specifically and uniquely breast cancer-focused in Department of Experimental Pathology program at Yale University with mentoring and training emphasis in breast cancer research that would not be possible without this grant.

Conclusion

The current research findings indicate that increased MAP-tau expression is associated with better outcome and that MAP-tau is a prognostic marker in early and metastatic breast cancer settings. However, we were not able to confirm the predictive value of MAP-tau as observed by the Pusztai group, a finding which may reflect differences in treatment settings since observations made by the Pusztai group occurred in the neoadjuvant setting only. The next phase of this project will examine additional microtubule related proteins multiplexed with MAP-tau. We hope to determine if specific combinations of microtubule stabilizing and destabilizing proteins, rather than simply one protein marker such as MAP-tau, may provide improved molecular signatures for taxane responsiveness.

Our findings may be reflective of increased mitotic arrest and inhibition of cellular proliferation within cancer cells that can occur when high levels of MAP-tau are present. Taxanes function in a similar manner to MAPs by binding and stabilizing microtubules leading to mitotic arrest in cancer cells. Thus, taxanes may be competing for binding sites with tau and this may explain why increased MAP-tau expression results in resistance to taxane treatment (lack of functional binding sites available for paclitaxel) and why low MAP-tau expression is predictive for paclitaxel response (abundance of functional binding sites available for paclitaxel).

The dual functionality of MAP-tau in combination with other MAPs may translate into increased tumor molecular screening information for patients with breast cancer resulting in better treatment options. Consequently, these microtubule associated proteins may serve as valuable biomarkers for the personalized molecular assessment of breast cancer tumors and we are working to systematically evaluate these proteins.

References

1. American Cancer Society. Cancer Facts and Figures 2007. Atlanta, GA, 2007
2.. Estevez, L.G. & Gradishar, W.J. Evidence-based use of neoadjuvant taxane in operable and inoperable breast cancer. *Clin Cancer Res* **10**, 3249-3261 (2004).
3. Lynch, H.T., Fusaro, R.M. & Lynch, J.F. Cancer genetics in the new era of molecular biology. *Annals of the New York Academy of Sciences* **833**, 1-28 (1997).
4. Riesterer, O., Milas, L. & Ang, K.K. Use of molecular biomarkers for predicting the response to radiotherapy with or without chemotherapy. *J Clin Oncol* **25**, 4075-4083 (2007).
5. Rouzier, R. *et al.* Microtubule-associated protein tau: a marker of paclitaxel sensitivity in breast cancer. *Proceedings of the National Academy of Sciences of the United States of America* **102**, 8315-8320 (2005).
6. US Cancer Statistics Working Group. United States cancer statistics: 1999--2002 incidence and mortality. Atlanta, GA: US Department of Health and Human Services, CDC, National Cancer Institute; 2005. Available at http://www.cdc.gov/cancer/npcr/uscs/index.htm.

Appendices

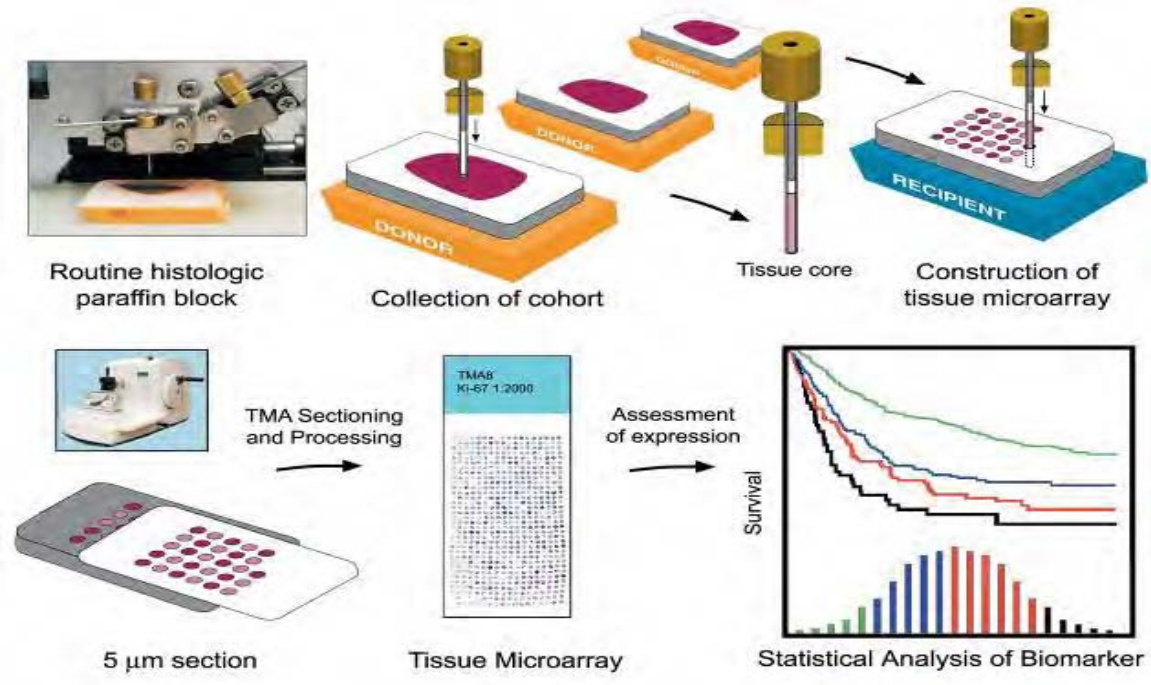
Appendix A

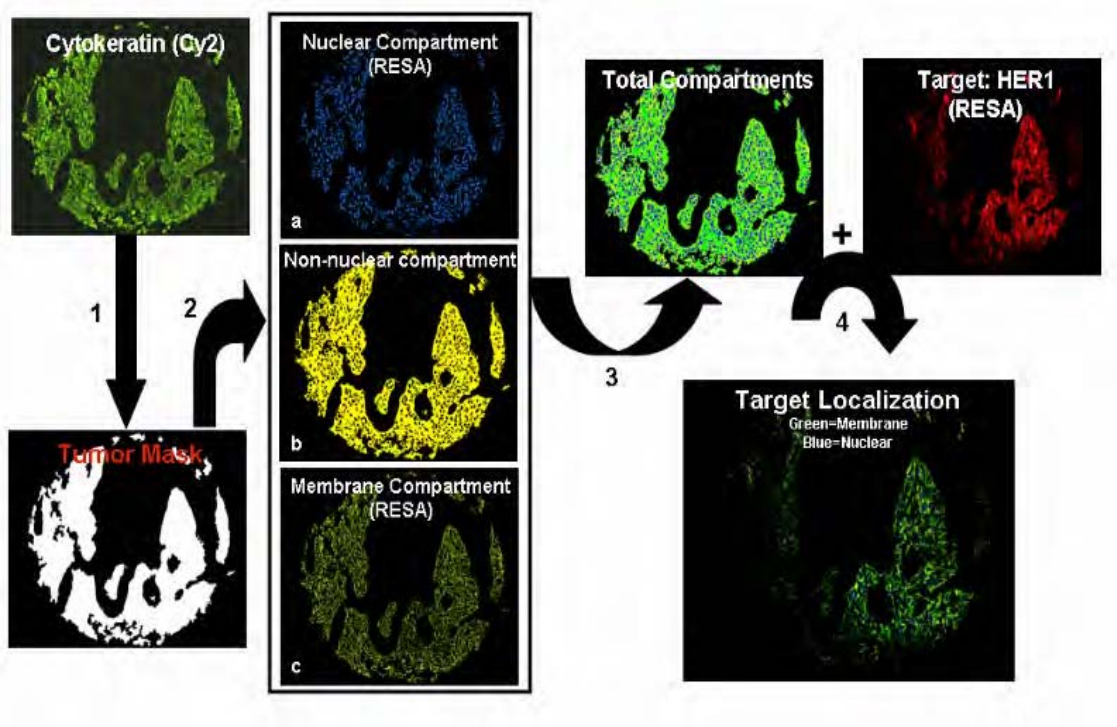
Automated Quantitative Analysis (AQUA)

What is an AQUA Score?

- Each pixel within the mask is assigned a user-defined subcellular compartment (or unassigned)
- The intensity of the "target" of interest is measured on a scale of 0-255 in each pixel in each compartment
- The final score is normalized by dividing the total target intensity by the area of each subcellular compartment.
- The final score is proportional to a number of molecules per unit area.

Methods and Instruments





AQUA Analysis of Tissue

The AQUA software linked to the fluorescence microscopy system allows for quantification
of the protein of interest within the tumor region of each tissue mircroarray core.

Step 1: Cytokeratin is used to separate epithelial tumor from surrounding stroma, creating a tumor mask

Step 2: Different fluorescent tags (like DAPI, Cy-5 tyramide) are used to demarcate subcellular compartments (nuclear, membrane, cytoplasmic, etc).

Step 3: Due to the thickness of the tissue sections and the resulting overlap of compartments, a rapid exponential subtraction algorithm (RESA) is used to subtract an out-of-focus image from an in-focus image, providing improved pixel assignment to subcellular compartments. An AQUA score is generated for each compartment ranging from 0-255 (see box *What is an AQUA score…)*

Step 4: At the Cy-5 wavelength, which is outside the range of tissue autofluroescence, the target of interest is tagged and measured within the subcelluar compartments by the PLACE algorithm.

The resulting AQUA score is the measurement of the biomarker pixel intensity within a compartment divided by the total area of the compartment (to normalize for differences in tumor area in each spot).

Appendix B
TAX 307 Patient Characteristics and Study Design

- Cases obtained from the TAX 306 Study Group (2003) (Dr. Lyndsay Harris, Yale Breast Cancer Center)
- Patients randomized to:
  1) FAC: 5-Fluorouracil + doxorubicin (DNA intercalation & anthracycline) + cyclophosphamide (alkylating agents) ; 56 patients total
  OR
  2) TAC: docetaxel + doxorubicin (DNA intercalation & anthracycline) + Cyclophosphamide (alkylating agent); 62 patients total

- Study Design:
  - Multicenter: 58 total in Europe, S. Africa, S. America
    Australia, Canada
  - Randomized (centralized)
  - Non-blinded
  - Phase III

- Primary endpoints: Time to treatment progression (TTP)

- Secondary endpoints: overall response rate (ORR), time to treatment failure (TTF), toxicity, survival, quality of life (QoL)

- Inclusion criteria:
  - adjuvant or neoadjuvant <u>non-anthracycline</u> chemo OK
  - prior hormonal therapy OK, but not concurrent
  - NO previous taxanes

- TAX 307 cohort: 140 cases from both TAC and FAC arms
- Censored:
  - Adverse events
  - Withdraw consent
  - Other reasons
  F=8 (14.2%), T=18 (29%)

  Uncensored:
  - Disease Progression
  - Max Number of cycles

Appendix C

TAX 307 Whole Tissue Sections
Methods

- 140 cases:
  - Floated, whole tumor sections
  - PLUS slides inconsistently used

- 85 matching H&E slides

- 6 control slides: YTMA 94-1; Cell lines for secondary normalization + staining quality control

- Staining:
  - 6 consecutive batches:
  - 25 slides/batch + 1 YTMA 94-1
  - 1 week period: early November 2006

- Target:
  - MAP-tau mouse monocolonal antibody
  - US Biological; 1:750 dilution (titrated)

- Image Capture:
  - HistoRx Image Grabber

- Quantitative analysis of specimens:
  - HistoRx AQUA

Appendix D

Title: MAP-tau expression is a prognostic marker but is not predictive in the metastatic setting in breast cancer.

Maria T. Baquero, MPH[1], Lyndsay Harris, MD. Mark Gustavson, PhD[1], Robert L. Camp, MD, PhD[1] and David L. Rimm, MD, PhD[1]. [1]Department of Pathology, Yale School of Medicine, New Haven, CT, 06520. Department of Clinical Oncology, Yale School of Medicine, New Haven, CT. 06520.

Background: Taxanes are microtubule targeting agents and potent cytotoxic molecules which have been recognized as highly effective chemotherapeutic agents. However, the response rates in the 40-60% range (depending on the setting) suggest the need for a companion diagnostic test with predictive value to determine patient sensitivity. One study has suggested that MAP-tau may serve as a companion diagnostic, but there are very few studies that have evaluated the prognostic or predictive value of MAP-tau in large patient cohorts.

Material and Methods: MAP-tau protein levels were measured using AQUA technology for quantitative assessment of protein expression on two cohorts. The training cohort consisted of a large retrospective breast cancer tissue microarray (n=480) with 20 year follow-up. This set was used to determine the optimal cut-point for MAP-tau prognostic value. The validation cohort was comprised of conventional slides from 140 cases of from the TAX 307 randomized clinical trial comparing FAC versus TAC treatment. AQUA scores were correlated with clinical and pathologic variables for TMA and whole sections. Cell line controls for both TMA and whole tumor sections were used to normalize AQUA scores and were highly correlated (R= 0.96).

Results: TMA analysis of the first cohort showed a normal distribution of expression with good reproducibility (R= 0.76 between paired TMA cores). Continuous univariate analysis indicated a protective relationship between MAP-tau expression and outcome (OR = 0.625, 95% confidence interval [CI] = 0.52-0.75; P<.0001). Optimal cut-point for binarization of the data was determined to be 12.4 units. The TAX307 trial, used as a validation set, showed significantly longer TTP for high expressers versus low expressers (Kaplan Meier analysis, median survival was 25 vs. 35.5 months; log rank, P = 0.0263). No significant differences were observed when MAP-tau was further stratified by treatment group TAC (n=56) vs FAC (n=62). Furthermore, no relationship was found between MAP-tau expression and response as measured by RECIST.

Discussion: This study shows that MAP-tau expression is a good prognostic marker with increased MAP-tau expression associated with improved 5 year

outcome and longer TTP. However, in contrast to previous results (Rouzier, R.*, et al., 2005*), MAP-tau expression was not found to be predictive of taxane response. Differences in patient populations may explain these contrasting results since Rouzier and colleagues evaluated MAP-tau expression in the neoadjuvant setting while this study evaluated MAP-tau in the metastatic setting. Since both studies are small trials, further work is required to assess the predictive value of MAP-tau. Additional MAPs, like Stathmin or other microtubule associated proteins may be required in order to predict taxane response in breast cancer patients.